Dimeric stilbene antibiotics target the bacterial cell wall in drug-resistant Gram-positive pathogens

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Materials and Methods

General Experimental Methods. Tapinarof was purchased from eNovation Chemicals LLC (Bridgewater Township, NJ). Resveratrol was purchased from TCI Chemicals (Tokyo, Japan). Pinosylvin was synthesized as previously described,[1] and duotap-520 and duotap-437 were purified as previously described.[2] Liquid chromatography-mass spectrometry was performed on an Agilent 1260 Infinity Series single quadrupole LC-MS using a Phenomenex Kinetix C18 column (4.6 x 250 mm). High-resolution mass spectrometry (HRMS) was performed on an Agilent iFunnel 6550 quadrupole time of flight (Q-TOF) MS instrument fitted with an electrospray ionization (ESI) source coupled to an Agilent 1290 Infinity HPLC system. Methicillin-resistant *Staphylococcus aureus* USA300 (ATCC strain BAA-1717) was used throughout the studies described; a clinical isolate of vancomycin-resistant *Enterococcus faecalis* obtained from Professor Martin Kriegel at Yale School of Medicine was used in the MIC studies.

Isolation and characterization of Duotap-468. Resveratrol (1 mM) was incubated in 100 mM sodium phosphate buffer (pH 7.4) supplemented with 1 mM $CuSO_4$ at 37 ^oC overnight. A total reaction volume of 200 mL was used. Following incubation, the reactions were frozen, lyophilized, and extracted with methanol to remove salts. Resveratrol and duotap-468 were separated on an Agilent 1260 Infinity LC system using a Phenomenex C18 column (10 x 250 mm, 2 mL/min, 10-60% aqueous acetonitrile with no acid for 30 minutes), and duotap-468 was collected at a retention time of 28.1 min. Collected fractions were frozen and dried by lyophilization. Nuclear magnetic resonance (NMR) characterization (^1H, gCOSY, gHSQCAD, and gHMBCAD) was performed on an Agilent 600 MHz NMR spectrometer with a cold probe. Purified duotap-468 was dissolved in 200 μL of MeOH-d_4. NMR spectra were analyzed using MestReNova software.

Minimal Inhibitory Concentration Assays. All compounds were prepared and used as stock solutions at a concentration of 10 mM in DMSO. Overnight cultures of each bacteria were grown at 37 °C. MRSA was grown in tryptic soy broth (TSB) medium and VRE was grown in brain heart infusion medium. Bacterial cultures were diluted to OD_{600} = 0.1, and 50 µl of diluted cell culture was added to each well of a 96 well plate with 50 µl of medium containing antibiotic at final concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 µM in triplicate. Plates were sealed and incubated overnight at 37 °C, and then OD_{600} was read using a PerkinElmer EnVision 2100 plate reader (PerkinElmer, Waltham, MA, USA). Minimal inhibitory concentrations for each compound were determined by fitting the data to a Gompertz model[3].

Modest Resistance Mutant Development. An overnight culture of MRSA was diluted 1:200 into TSB medium containing antibiotic at 0.5X MIC and 4X MIC and incubated at 37 °C overnight. Bacterial cultures from the highest concentration that showed growth (OD_{600} > 0.1) were used to inoculate cultures the next day. When bacterial growth was observed at the highest antibiotic concentration used, cultures were re-inoculated at successively higher concentrations (4X MIC, 8X MIC, 12X MIC, etc.). For example, when growth of bacteria was first observed in the presence of antibiotic at 4X MIC, this culture was re-inoculated at 0.5X, 4X, and 8X MIC to encourage further resistance development. Two independent cultures were grown in the presence of duotap-520 for 90 days. One culture was grown in the presence of ciprofloxacin for 30 days as a positive control for development of antibiotic resistance. The fold change in MIC for resistant cultures was assessed at 30, 60, and 90 days. The resistance cultures were streaked out on LB plates, and three independent colonies were inoculated in TSB. The MIC for each was determined as described above, and fold change in MIC was determined by comparison to the MIC of a day 0 wildtype MRSA culture.

Resistance Mutant Genome Sequencing. Resistance cultures at days 0, 30, 60, and 90 were streaked on LB plates and grown at 37 °C overnight. The next day, individual colonies were inoculated in TSB (5 mL) and grown overnight at 37 °C with shaking. High molecular weight genomic DNA was prepared from each culture using phenol-chloroform extraction. Cells were pelleted by centrifugation and re-suspended in 1 mL of extraction buffer (100 mM Tris pH 8, 100 mM EDTA pH 8, 1.4 M NaCl, 1% CTAB). Lysozyme (5 mg) and 1 µL of 100 mg/mL RNaseA were added to each extraction, and extractions were incubated at 37 °C for 20 min. To each extraction, SDS (100 µL of a 10% solution) and 10 µL of 10 mg/mL proteinase K were added. Extractions were then incubated at 56 °C for 1 h and centrifuged for 5 min at 1600 x *g*. The supernatant of each extraction was transferred to a new tube and the volume of each was adjusted to 2 mL with 10 mM Tris (pH 8). A mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was prepared, and 1.5 mL of this mixture was added to each extraction. The organic and aqueous layers were mixed and then separated by centrifugation for 5 min at 1600 x *g*. The aqueous layer was washed twice with 1.5 mL of chloroform, and gDNA was purified from the aqueous layer by isopropanol precipitation.

Samples were submitted for sequencing at the Yale Center for Genome Analysis using an Illumina HiSeq 4000. Illumina paired-end reads were trimmed and subsequently mapped to the *Staphylococcus aureus subsp. aureus USA300_TCH1516* reference genome (Genbank: CP000730.1) using Bowtie2. The --fast-local preset options were used for the alignment. Average coverage was 167. Aligned reads were visualized with Geneious 8.1.9. Variants were called with the following parameters: variant frequency >25%; Variant P-value <10E-6; Strand Bias P-value >10E-5 when exceeding 65% bias.

MRSA Membrane Integrity Assay. The effects of duotap on MRSA membrane permeability were assessed using SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific). MRSA was inoculated in TSB and grown to an OD_{600} of 0.35. Cells were washed with phosphate buffered saline (PBS) three times and resuspended in PBS at an OD_{600} of 0.35. Cells (900 μL) were mixed with 100 μL of 17 μM SYTOX Green and incubated for 5 min at room temperature. The mixture of cells and dye was then distributed into a 96 well plate (50 μL per well) and fluorescence was measured at excitation and emission wavelengths of 488 nm and 523 nm, respectively, on a Perkin Elmer EnVision plate reader. Into each well, 50 μL of PBS containing antibiotic was added (giving a final antibiotic concentration of 20 μg/mL),[4] and fluorescence measurements were immediately collected for 14 min. Vancomycin was used as a negative control, and daptomycin was used as a positive control. In the daptomycin-containing wells, 15 mM $CaCl_2$ was supplemented into all media used. All antibiotics were tested in three replicates.

Cell Wall Precursor Accumulation Assay. MRSA was inoculated in TSB and grown to an OD_{600} of 0.6. Chloramphenicol (130 μg/mL) was added to 1 mL of culture and incubated at 37 ^{o}C for 15 min. Duotap or vancomycin was then added at a concentration of 2.5 times the MIC for each compound, and the cultures were incubated at 37 ^{o}C for 60 min. Cells were pelleted by centrifugation, resuspended in 180 μL of dH_2O and 20 μL of 100 mM ammonium acetate pH 4.2 (to promote removal of lipid II tail)[5], and incubated at 95 ^{o}C for 30 min. The cell extract was centrifuged, and the supernatant was frozen, lyophilized, and resuspended in methanol. Samples were analyzed for accumulation of the UDP-MurNAc-pentapeptide and de-lipidated lipid II cell wall precursors compared to DMSO negative control by Q-TOF LC-MS. All experiments were performed in triplicate. Tandem mass spectrometry was also performed to verify the identity of de-lipidated lipid II with a collision energy of 50 V.

Lipid II Complementation Assay. An overnight culture of MRSA was grown at 37 °C in TSB. The culture was diluted to an OD_{600} of 0.1, and 50 μl of diluted cell culture was added to each well of a 96 well plate. TSB medium (50 μL) containing antibiotic at twice the MIC and two molar equivalents of lipid II (or an equal volume of DMSO as a control) was also added to each well. Plates were sealed and incubated overnight at 37 °C, and then the OD_{600} was read. All conditions were tested in triplicate. Lipid II isolated from MRSA was used as a 50 μM solution in DMSO.

MTT Assay for Cell Viability. Human colon (HCT116) cells (10,000) were inoculated into each well of a 96 well plate (Costar 3595) in 100 uL DMEM/F12 medium containing 5% heat-inactivated FBS + 25 mM HEPES. Cells were incubated at 37 °C in 5% CO_2. After 24 hours, compounds were added to appropriate wells while maintaining a fixed 1% final vehicle DMSO concentration. Cells were incubated with compound for an additional 72 hours. Cell viability was quantified with Promega's CellTiter Glo reagent using the standard manufacturer's protocol. Luminescence was measured on a Perkin Elmer EnVision 2100 plate reader with a 1s exposure time.

Supporting Figures

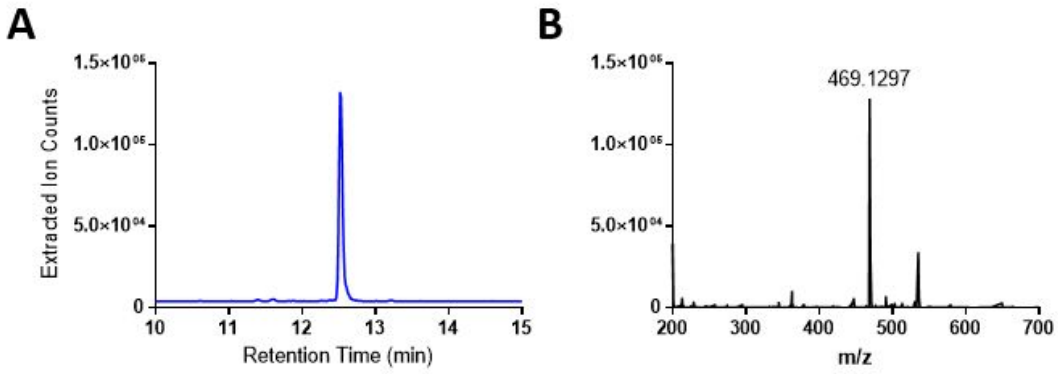

Figure S1. Extracted ion chromatogram (EIC) (**A**) and HR-ESI-QTOF-MS (**B**) for duotap-468.

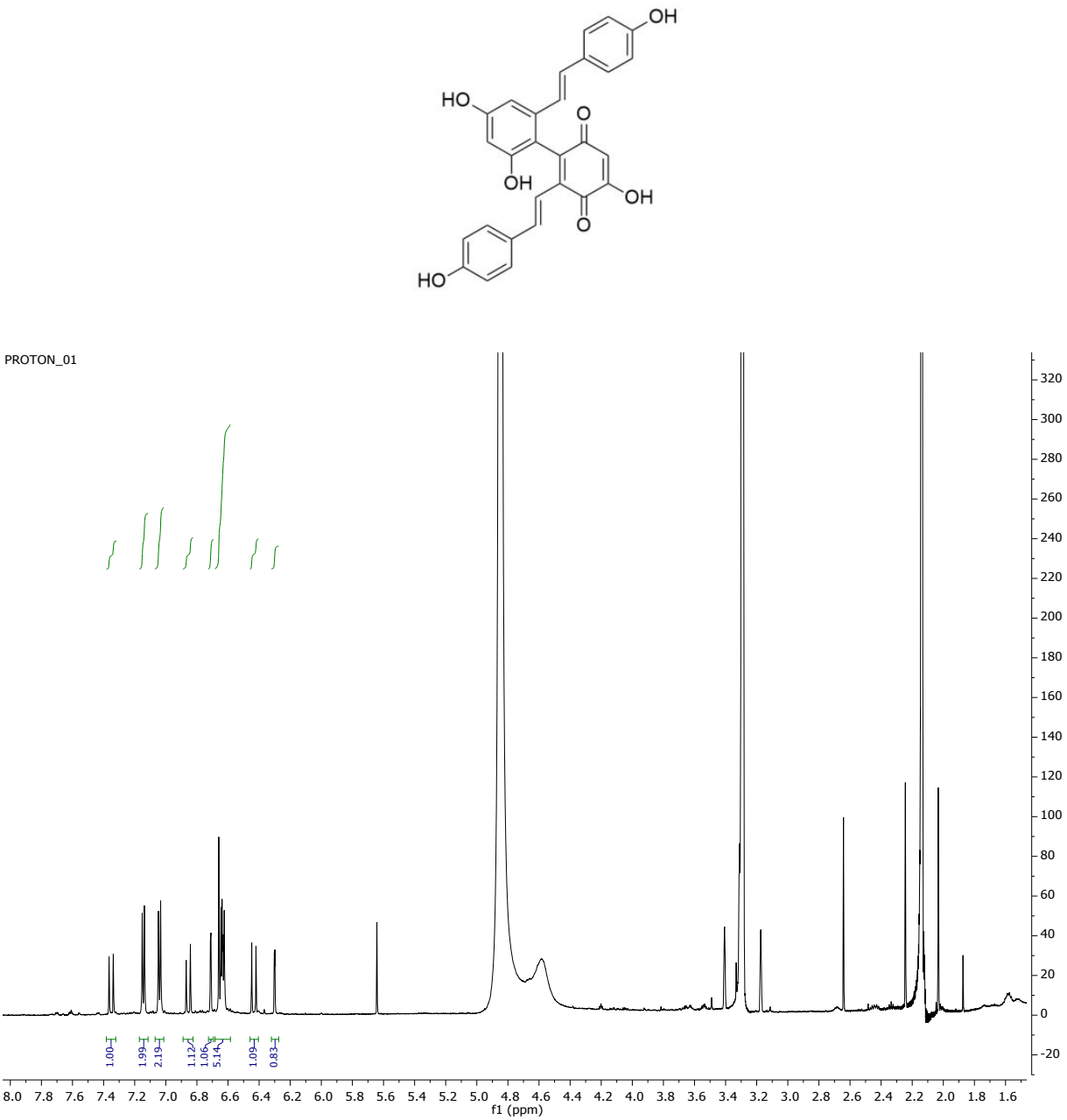

Figure S2. ^{1}H NMR spectrum of duotap-468 in methanol-d_4.

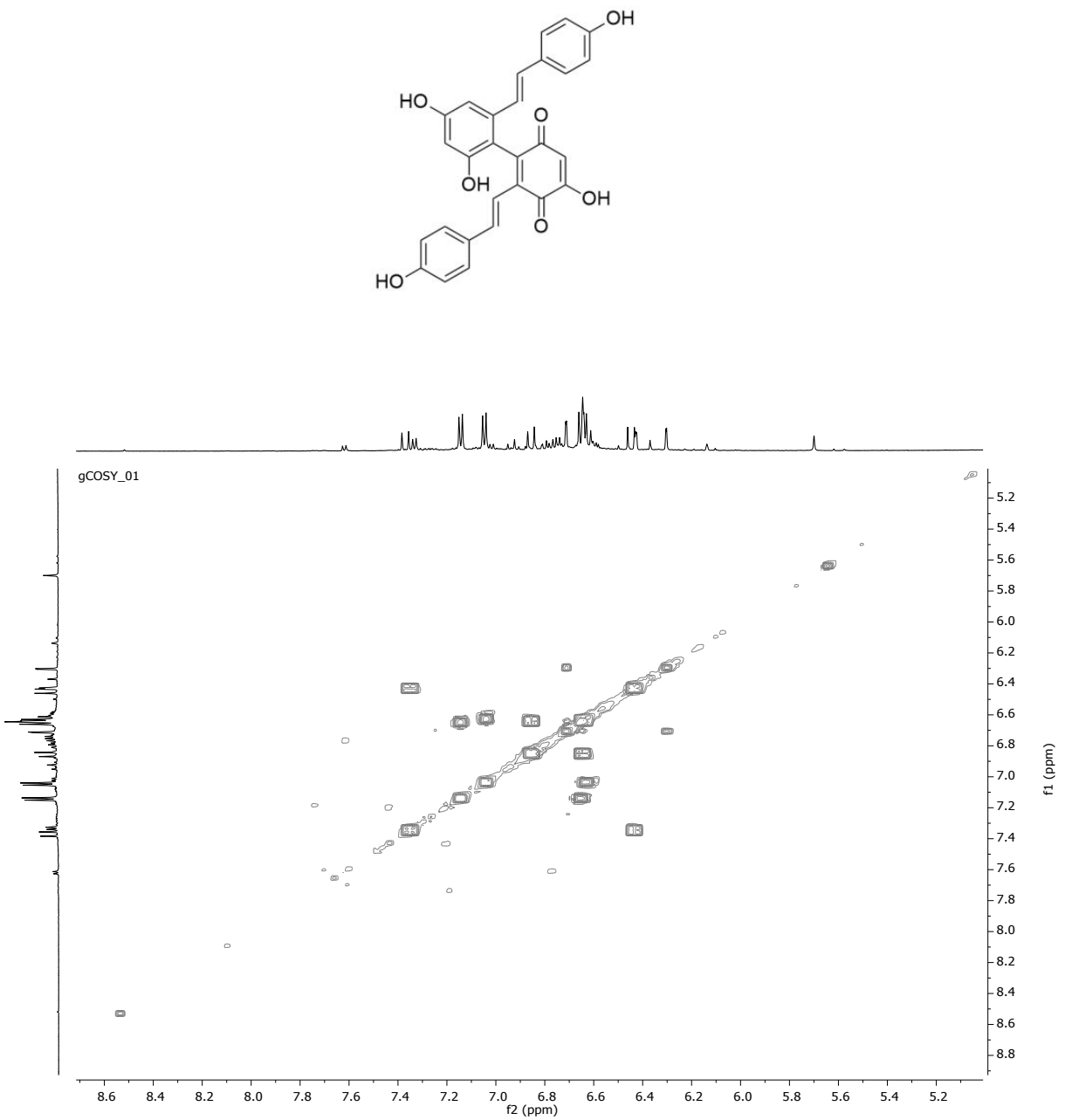

Figure S3. gCOSYAD NMR spectrum of duotap-468 in methanol-d_4.

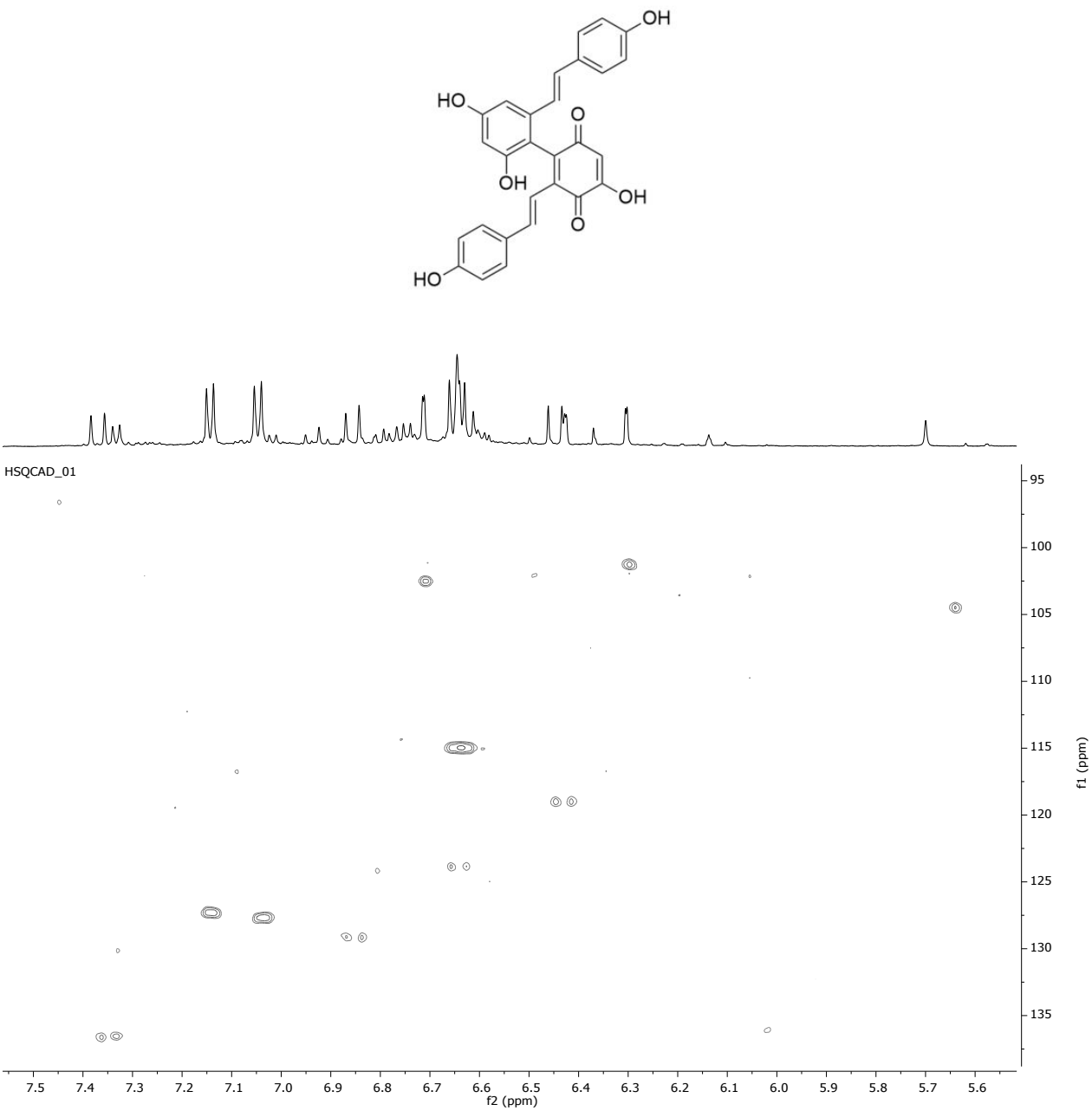

Figure S4. gHSQCAD NMR spectrum of duotap-468 in methanol-d_4.

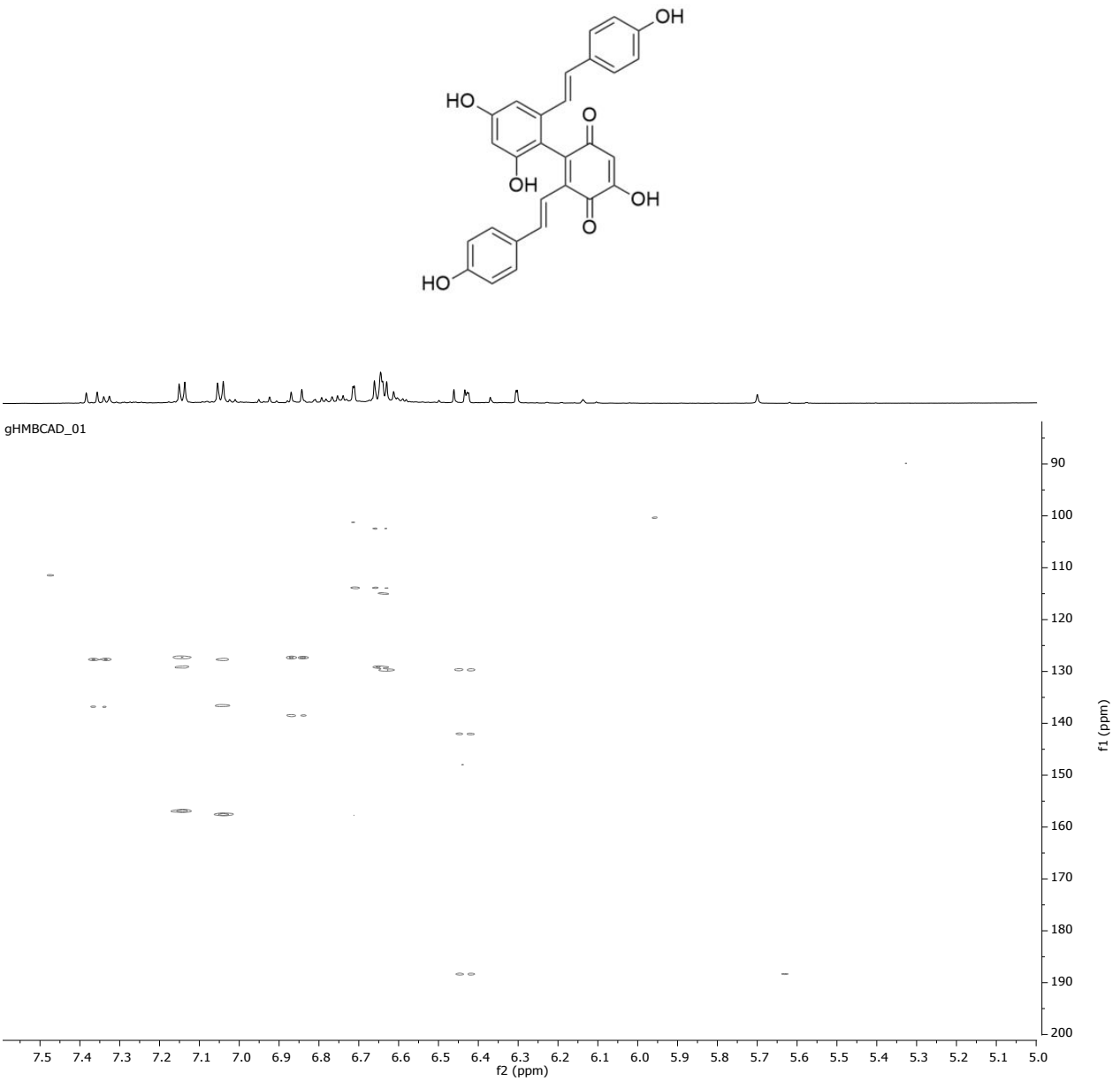

Figure S5. gHMBCAD NMR spectrum of duotap-468 in methanol-d_4.

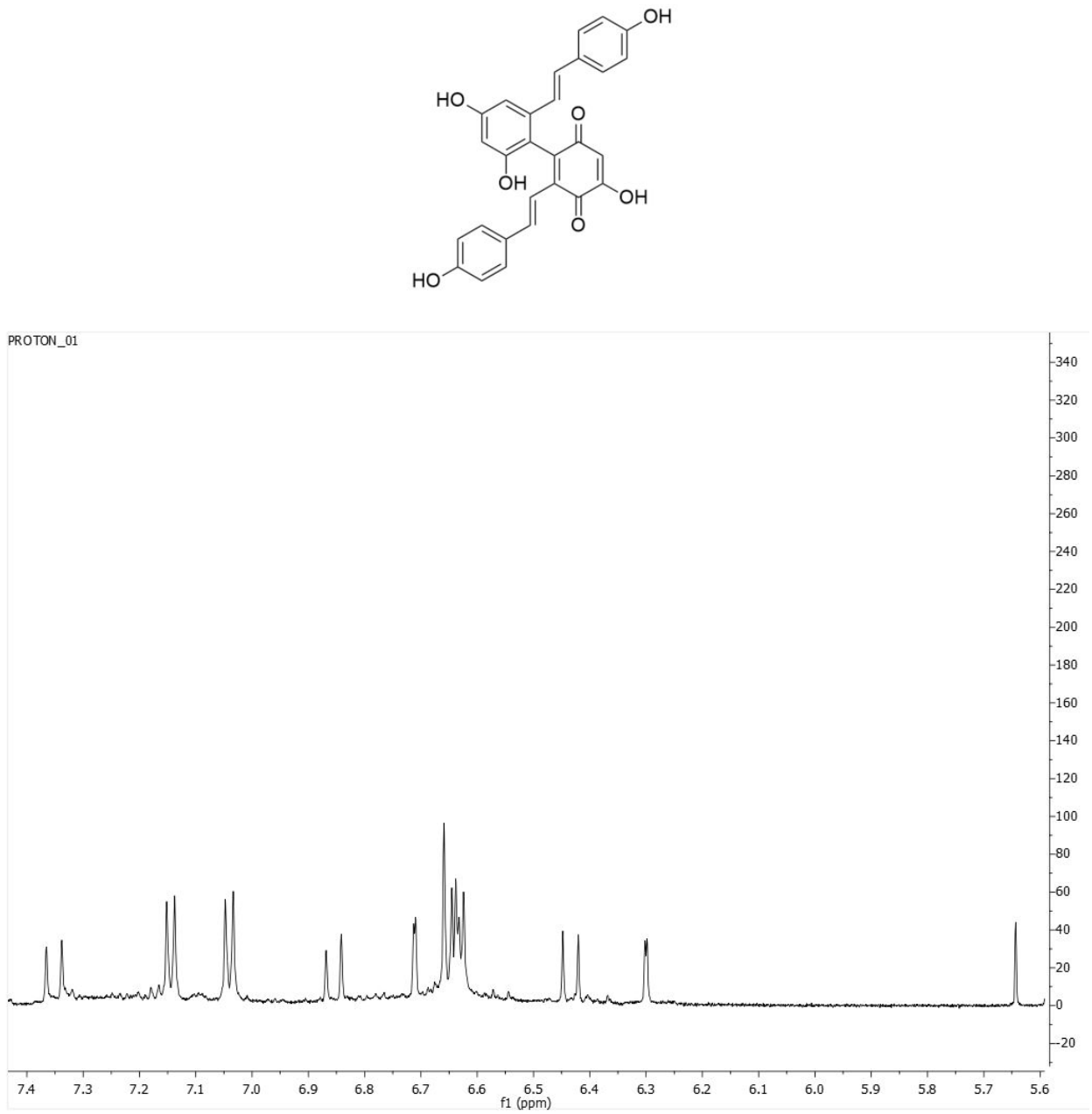

Figure S6. Confirmation of duotap-468 purity after NMR characterization and drying by ^{1}H NMR in methanol-d_4.

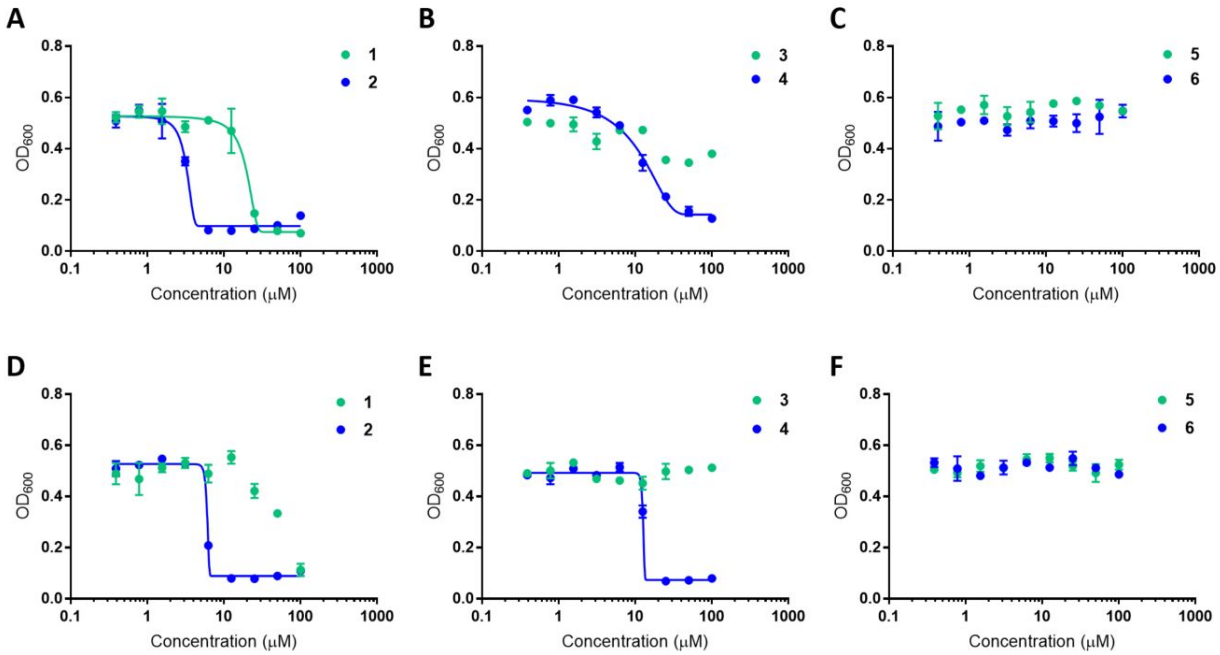

Figure S7. Effects of stilbene monomer to dimer conversion on the minimal inhibitory concentrations (MICs) of compounds **1-6** against methicillin-resistant *Staphylococcus aureus* (MRSA, A-C) and vancomycin-resistant *Enterococcus faecalis* (VRE, D-F). Data is represented in triplicate with error bars showing the standard deviation.

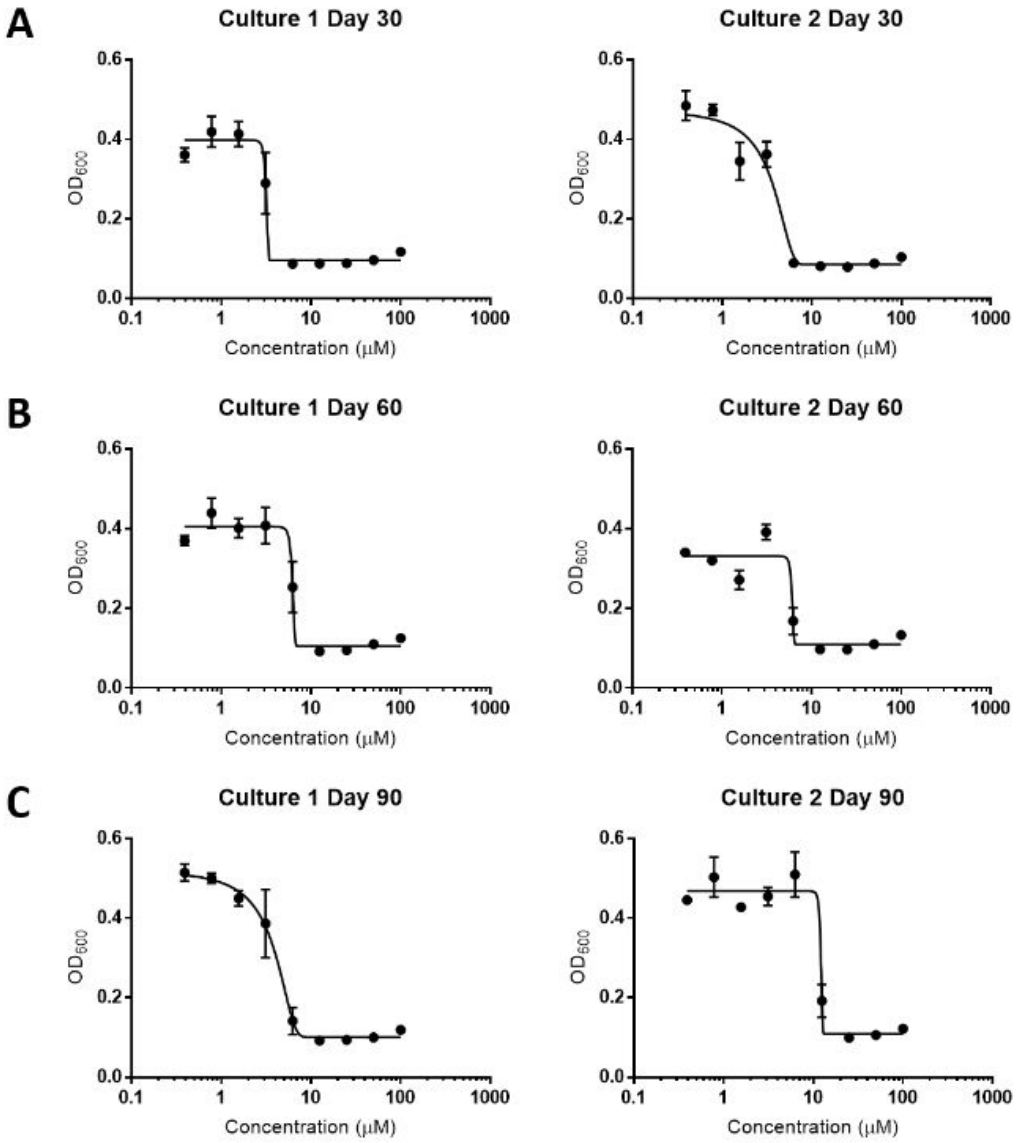

Figure S8. Growth inhibitory effects of duotap-520 against two independent cultures of methicillin-resistant *Staphylococcus aureus* (MRSA) after growth in the presence of sub-inhibitory levels of antibiotic for 30 days (**A**), 60 days (**B**), and 90 days (**C**). Data is represented in triplicate with error bars showing the standard deviation. The MIC for each culture was tested three times, and representative data are shown.

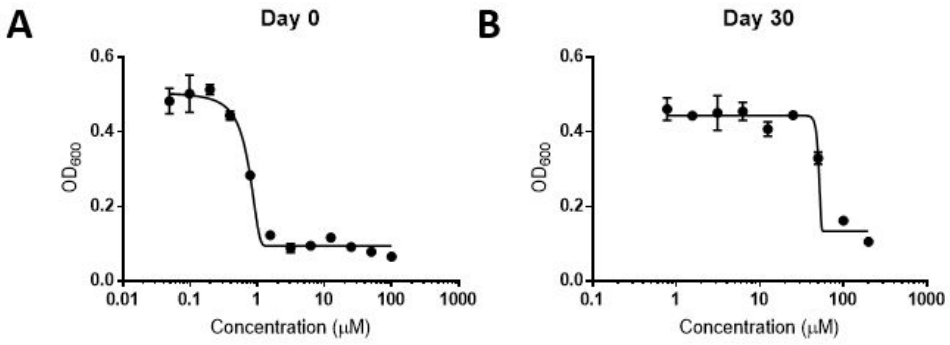

Figure S9. Growth inhibitory effects of ciprofloxacin against methicillin-resistant *Staphylococcus aureus* (MRSA) after growth in the presence of sub-inhibitory levels of ciprofloxacin for 0 days (**A**) and 30 days (**B**). Data is represented in triplicate with error bars showing the standard deviation. The MIC for each culture was tested three times, and representative data are shown.

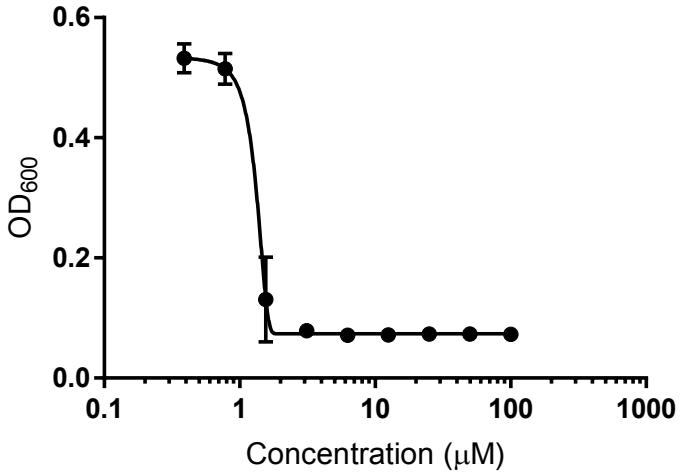

Figure S10. Growth inhibitory effects of vancomycin against MRSA.

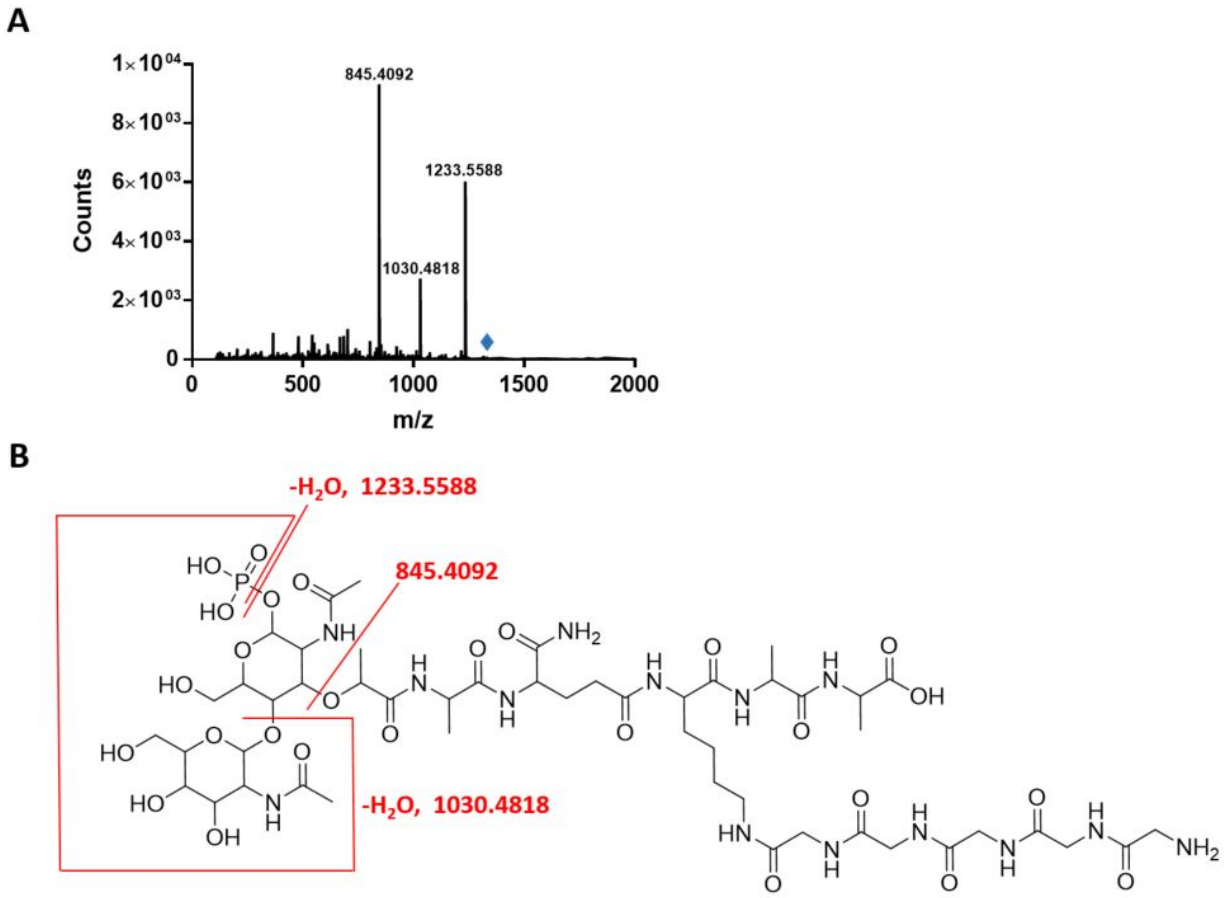

Figure S11. Tandem mass spectrum of de-lipidated lipid II $[M+H]^+$ = 1331.5362 (**A**) and structures of fragment masses observed (**B**).

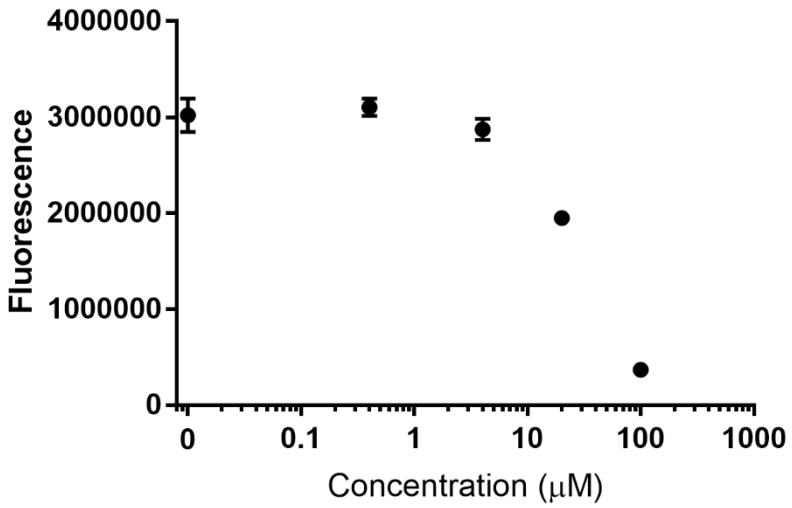

Figure S12. MTT assay for human colon (HCT116) cell viability in the presence of duotap-520. Noticeable toxicity was observed at 20 μM, and cell growth was completely inhibited at 100 μM. The MIC of duotap-520 against MRSA is 4 μM.

Supporting Tables

Table S1. Minimal inhibitory concentrations (μM) of compounds **1-6** against MRSA and VRE.

	S. aureus (methicillin-resistant)	*E. faecalis* (vancomycin-resistant)
1	27	51
2	4	6
3	>100	>100
4	25	14
5	>100	>100
6	>100	>100

Table S2. Mutations in MRSA at Day 0 of Subculturing

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,915	T -> C		SNP	28.60%	
2,872,914	A -> C		SNP	29.00%	
2,872,914	A -> G		SNP	29.00%	
2,872,912	TT -> AC		Substitution	29.3% -> 32.4%	
1,419,999	G -> A	V -> I	SNP	36.50%	M3 family oligoendopeptidase F
512,874	C -> T		SNP	26.60%	
512,643	A -> G		SNP	37.20%	
111,516	C -> A	Q -> K	SNP	37.60%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	40.50%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	43.30%	
76,322	G -> A		SNP	39.80%	
76,295	T -> C		SNP	47.00%	
76,265	C -> T		SNP	28.00%	
36,608	G -> T		SNP	32.50%	
36,581	C -> T		SNP	44.30%	
36,479	T -> C	T -> A	SNP	60.10%	IS431mec transposase
36,471	A -> G		SNP	40.40%	IS431mec transposase
36,444	C -> T		SNP	43.90%	IS431mec transposase
36,413	C -> T	V -> I	SNP	43.50%	IS431mec transposase
36,315	G -> A		SNP	58.00%	IS431mec transposase
36,269	C -> T	A -> T	SNP	57.70%	IS431mec transposase
36,124	G -> A	A -> V	SNP	54.10%	IS431mec transposase
35,817	A -> T		SNP	36.30%	
35,816	A -> C		SNP	25.90%	
4	A -> T		SNP	28.60%	
2	(ACT)2 -> (ACT)3		Insertion	25.00%	
2	C -> T		SNP	75.00%	
1	A -> T		SNP	37.50%	

Table S3. Mutations in MRSA Culture 1 at Day 30 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,915	T -> C		SNP	35.00%	
2,872,914	A -> C		SNP	42.90%	
2,872,913	T -> A		SNP	27.30%	
2,872,911	+TA		Insertion	29.40%	
2,872,911	(T)4 -> (T)5		Insertion	35.30%	
2,872,911	(T)4 -> (T)5		Insertion	26.50%	
2,149,327	G -> T	A -> S	SNP	25.90%	accessory gene regulator protein C
1,419,999	G -> A	V -> I	SNP	31.00%	M3 family oligoendopeptidase F
512,643	A -> G		SNP	30.50%	
111,516	C -> A	Q -> K	SNP	34.40%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	36.80%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	44.70%	
76,322	G -> A		SNP	44.30%	
76,295	T -> C		SNP	51.20%	
76,265	C -> T		SNP	25.50%	
36,608	G -> T		SNP	30.80%	
36,581	C -> T		SNP	46.40%	
36,479	T -> C	T -> A	SNP	53.10%	IS431mec transposase
36,471	A -> G		SNP	35.10%	IS431mec transposase
36,444	C -> T		SNP	39.10%	IS431mec transposase
36,413	C -> T	V -> I	SNP	41.50%	IS431mec transposase
36,315	G -> A		SNP	58.50%	IS431mec transposase
36,269	C -> T	A -> T	SNP	57.50%	IS431mec transposase
36,124	G -> A	A -> V	SNP	58.10%	IS431mec transposase
36,087	A -> G		SNP	56.40%	IS431mec transposase
35,816	A -> C		SNP	26.90%	
4	+ATACTA		Insertion	25.00%	
4	A -> T		SNP	25.00%	
2	(ACT)2 -> (ACT)3		Insertion	42.30%	
2	C -> A		SNP	34.60%	
2	C -> T		SNP	50.00%	

Table S4. Mutations in MRSA Culture 2 at Day 30 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,914	A -> C		SNP	33.30%	
2,872,911	(T)4 -> (T)5		Insertion	29.00%	
2,149,327	G -> T	A -> S	SNP	31.80%	accessory gene regulator protein C
1,419,999	G -> A	V -> I	SNP	32.00%	M3 family oligoendopeptidase F
992,936	G -> T	V -> F	SNP	27.20%	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
512,643	A -> G		SNP	30.40%	
111,516	C -> A	Q -> K	SNP	32.20%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	31.90%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	43.70%	
76,322	G -> A		SNP	40.90%	
76,295	T -> C		SNP	51.10%	
76,265	C -> T		SNP	26.10%	
36,608	G -> T		SNP	29.60%	
36,581	C -> T		SNP	50.70%	
36,479	T -> C	T -> A	SNP	60.20%	IS431mec transposase
36,471	A -> G		SNP	35.70%	IS431mec transposase
36,444	C -> T		SNP	42.50%	IS431mec transposase
36,413	C -> T	V -> I	SNP	46.80%	IS431mec transposase
36,315	G -> A		SNP	55.40%	IS431mec transposase
36,269	C -> T	A -> T	SNP	53.90%	IS431mec transposase
36,124	G -> A	A -> V	SNP	59.00%	IS431mec transposase
2	(ACT)2 -> (ACT)3		Insertion	40.00%	
2	C -> A		SNP	28.00%	
2	C -> T		SNP	28.00%	
1	A -> T		SNP	31.60%	

Table S5. Mutations in MRSA Culture 1 at Day 60 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,915	T -> C		SNP	60.00%	
2,872,912	T -> C		SNP	31.40%	
2,149,327	G -> T	A -> S	SNP	76.50%	accessory gene regulator protein C
2,149,325	A -> T	N -> I	SNP	60.60%	accessory gene regulator protein C
2,149,322	+T		Insertion	33.30%	accessory gene regulator protein C
2,149,319	T -> G	L -> R	SNP	42.10%	accessory gene regulator protein C
2,149,317	T -> A		SNP	38.90%	accessory gene regulator protein C
2,149,110	AAT -> GGC	N -> G	Substitution	27.6% -> 28.6%	accessory gene regulator protein C
2,149,106	AA -> TG		Substitution	33.3% -> 36.1%	accessory gene regulator protein C
1,274,154	C -> T	A -> V	SNP	47.20%	CodY family transcriptional regulator
992,936	G -> T	V -> F	SNP	26.20%	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein
736,885	(A)2 -> (A)3		Insertion	38.90%	ABC superfamily ATP binding cassette transporter, membrane protein (vraE)
512,874	C -> T		SNP	25.30%	
512,643	A -> G		SNP	35.30%	
111,516	C -> A	Q -> K	SNP	34.60%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	34.00%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	42.10%	
76,322	G -> A		SNP	39.10%	
76,295	T -> C		SNP	46.50%	
76,265	C -> T		SNP	26.70%	
36,581	C -> T		SNP	47.90%	
36,479	T -> C	T -> A	SNP	46.30%	IS431mec transposase
36,471	A -> G		SNP	27.50%	IS431mec transposase
36,413	C -> T	V -> I	SNP	41.40%	IS431mec transposase
36,315	G -> A		SNP	53.90%	IS431mec transposase
36,269	C -> T	A -> T	SNP	55.10%	IS431mec transposase

36,124	G -> A	A -> V	SNP	55.10%	IS431mec transposase
36,087	A -> G		SNP	57.60%	IS431mec transposase
35,817	A -> T		SNP	30.70%	
2	C -> A		SNP	37.50%	
2	C -> T		SNP	41.70%	
2	(ACT)2 -> (ACT)3		Insertion	50.00%	
1	A -> T		SNP	64.70%	

Table S6. Mutations in MRSA Culture 2 at Day 60 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,914	A -> C		SNP	52.20%	
2,872,913	T -> A		SNP	34.60%	
2,872,911	(T)4 -> (T)5		Insertion	32.10%	
2,872,911	(T)4 -> (T)5		Insertion	25.00%	
2,872,911	(T)4 -> (T)5		Insertion	42.90%	
2,872,911	+AC		Insertion	32.10%	
2,149,327	G -> T	A -> S	SNP	61.10%	accessory gene regulator protein C
2,149,325	A -> T	N -> I	SNP	41.30%	accessory gene regulator protein C
2,149,322	+T		Insertion	29.30%	accessory gene regulator protein C
2,149,319	T -> G	L -> R	SNP	36.40%	accessory gene regulator protein C
2,149,110	T -> C		SNP	26.90%	accessory gene regulator protein C
2,149,104	+GC		Insertion	26.20%	accessory gene regulator protein C
1,758,858	(C)3 -> (C)4		Insertion	34.80%	hypothetical protein
736,885	(A)2 -> (A)3		Insertion	29.90%	ABC superfamily ATP binding cassette transporter, membrane protein (vraE)
512,874	C -> T		SNP	27.50%	
512,643	A -> G		SNP	31.80%	
156,714	G -> A		SNP	36.60%	transcriptional regulator
111,516	C -> A	Q -> K	SNP	32.90%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	34.20%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	43.60%	
76,322	G -> A		SNP	41.20%	
76,295	T -> C		SNP	49.30%	
76,265	C -> T		SNP	25.20%	
36,608	G -> T		SNP	31.30%	
36,581	C -> T		SNP	46.20%	
36,479	T -> C	T -> A	SNP	57.70%	IS431mec transposase
36,471	A -> G		SNP	39.20%	IS431mec transposase
36,444	C -> T		SNP	43.00%	IS431mec transposase

36,413	C -> T	V -> I	SNP	44.30%	IS431mec transposase
36,315	G -> A		SNP	55.80%	IS431mec transposase
36,269	C -> T	A -> T	SNP	53.90%	IS431mec transposase
36,124	G -> A	A -> V	SNP	57.80%	IS431mec transposase
36,087	A -> G		SNP	56.60%	IS431mec transposase
2	C -> A		SNP	40.90%	
2	C -> T		SNP	27.30%	
2	(ACT)2 -> (ACT)3		Insertion	50.00%	
1	A -> T		SNP	64.70%	

Table S7. Mutations in MRSA Culture 1 at Day 90 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,915	T -> C		SNP	37.50%	
2,872,914	A -> C		SNP	36.40%	
2,872,911	(T)4 -> (T)5		Insertion	29.30%	
2,357,993	-TAATCC	GL ->	Deletion	28.5% -> 29.1%	DNA-directed RNA polymerase alpha subunit
2,149,327	G -> T	A -> S	SNP	100.00%	accessory gene regulator protein C
2,149,325	A -> T	N -> I	SNP	72.70%	accessory gene regulator protein C
2,149,324	A -> T	N -> Y	SNP	32.40%	accessory gene regulator protein C
2,149,322	+T		Insertion	50.00%	accessory gene regulator protein C
2,149,319	T -> G	L -> R	SNP	83.30%	accessory gene regulator protein C
2,149,317	ATT -> CTA	I -> L	Substitution	72.7% -> 100.0%	accessory gene regulator protein C
2,149,110	AAT -> GGC	N -> G	Substitution	40.9% -> 42.1%	accessory gene regulator protein C
2,149,106	AA -> TG		Substitution	46.2% -> 51.4%	accessory gene regulator protein C
2,149,104	+GC		Insertion	28.20%	accessory gene regulator protein C
2,128,358	G -> A		SNP	25.20%	
1,759,187	C -> T		SNP	46.70%	hypothetical protein
992,936	G -> T	V -> F	SNP	99.40%	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein (oppA1)
909,762	T -> A		SNP	26.40%	
909,758	A -> T		SNP	25.30%	
734,971	(A)7 -> (A)6		Deletion	86.30%	possible ABC superfamily ATP binding cassette transporter, ABC protein (vraF)
512,874	C -> T		SNP	25.80%	
512,643	A -> G		SNP	29.40%	
236,819	A -> G	I -> V	SNP	44.10%	oligopeptide ABC superfamily ATP binding cassette transporter, membrane protein

111,516	C -> A	Q -> K	SNP	33.60%	possible staphylococcal tandem lipoprotein
111,512	A -> G		SNP	36.60%	possible staphylococcal tandem lipoprotein
76,353	G -> A		SNP	41.10%	
76,322	G -> A		SNP	40.20%	
76,295	T -> C		SNP	49.10%	
76,265	C -> T		SNP	25.20%	
36,581	C -> T		SNP	47.30%	
36,479	T -> C	T -> A	SNP	51.20%	IS431mec transposase
36,471	A -> G		SNP	35.80%	IS431mec transposase
36,444	C -> T		SNP	38.70%	IS431mec transposase
36,413	C -> T	V -> I	SNP	42.10%	IS431mec transposase
36,315	G -> A		SNP	55.10%	IS431mec transposase
36,269	C -> T	A -> T	SNP	56.30%	IS431mec transposase
36,124	G -> A	A -> V	SNP	53.90%	IS431mec transposase
36,087	A -> G		SNP	55.00%	IS431mec transposase
26,710	A -> G	I -> V	SNP	42.70%	sensor histidine kinase VicK
4	A -> T		SNP	29.70%	
4	+ATACTA		Insertion	29.70%	
2	C -> A		SNP	42.30%	
2	C -> T		SNP	42.30%	
2	(ACT)2 -> (ACT)3		Insertion	38.50%	
1	A -> T		SNP	63.20%	

Table S8. Mutations in MRSA Culture 2 at Day 90 of Subculturing with Duotap

Position	Base change	Amino acid change	Polymorphism type	Variant frequency	Annotated gene product
2,872,915	T -> C		SNP	25.00%	
2,872,914	A -> C		SNP	36.00%	
2,872,911	(T)4 -> (T)5		Insertion	30.00%	
2,692,688	G -> T	GL ->	SNP	99.50%	
2,149,327	G -> T	A -> S	SNP	100.00%	accessory gene regulator protein C
2,149,325	A -> T	N -> I	SNP	73.10%	accessory gene regulator protein C
2,149,324	A -> T	N -> Y	SNP	31.80%	accessory gene regulator protein C
2,149,322	+T		Insertion	65.00%	accessory gene regulator protein C
2,149,319	T -> G	L -> R	SNP	87.50%	accessory gene regulator protein C
2,149,317	ATT -> CTA	I -> L	Substitution	80.0% -> 100.0%	accessory gene regulator protein C
2,149,110	AAT -> GGC	N -> G	Substitution	40.0% -> 44.4%	accessory gene regulator protein C
2,149,106	AA -> TG		Substitution	57.7% -> 62.1%	accessory gene regulator protein C
2,149,104	+GC		Insertion	27.60%	accessory gene regulator protein C
1,106,272	G -> A		SNP	84.90%	dihydrolipoyl dehydrogenase
1,005,832	-TTTGA		Deletion	64.2% -> 65.4%	possible dithiol-disulfide isomerase
992,936	G -> T	V -> F	SNP	99.20%	oligopeptide ABC superfamily ATP binding cassette transporter, binding protein (oppA1)
734,971	(A)7 -> (A)6		Deletion	88.10%	possible ABC superfamily ATP binding cassette transporter, ABC protein (vraF)
512,643	A -> G		SNP	34.30%	
218,120	C -> A		SNP	88.40%	possible LIVCS family branched chain amino acid:cation symporter
111,516	C -> A		SNP	29.70%	possible staphylococcal tandem lipoprotein

111,512	A -> G		SNP	30.80%	possible staphylococcal tandem lipoprotein
76,353	G -> A	I -> V	SNP	45.70%	
76,322	G -> A	Q -> K	SNP	41.30%	
76,295	T -> C		SNP	46.30%	
76,265	C -> T		SNP	29.30%	
36,581	C -> T		SNP	51.70%	
36,479	T -> C		SNP	55.40%	IS431mec transposase
36,471	A -> G		SNP	34.40%	IS431mec transposase
36,444	C -> T		SNP	40.00%	IS431mec transposase
36,413	C -> T	T -> A	SNP	44.90%	IS431mec transposase
36,315	G -> A		SNP	57.50%	IS431mec transposase
36,269	C -> T		SNP	58.40%	IS431mec transposase
36,124	G -> A	V -> I	SNP	60.10%	IS431mec transposase
36,087	A -> G		SNP	57.80%	IS431mec transposase
4	A -> T	A -> T	SNP	35.70%	
4	+T	A -> V	Insertion	25.00%	
4	-ATACTA		Insertion	35.70%	
2	C -> A	I -> V	SNP	52.20%	
2	C -> T		SNP	30.40%	
2	(ACT)2 -> (ACT)3		Insertion	39.10%	
1	A -> T		SNP	50.00%	

Supporting References

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